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(54) Title: IN VITRO FLUID TOXICITY BIOASSAY

(57) Abstract

A method for bioassaying fluid potentially containing toxicants is provided by the present disclosure. The method comprises contacting a plurality of compositions with the fluid and detecting at least one change, to obtain a toxicant profile. Also provided is a kit to practice the method. The method identifies toxicants or toxicant classes by means of a toxicity profile based on multiple bioassays.

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IN VITRO FLUID TOXICITY BIOASSAY

Technical Field

This invention relates to the field of testing fluids potentially containing toxicants. More specifically, the invention relates to a device and a method for an *in vitro* test which can determine the biological toxicity and toxicity profile of environmental fluid samples such as waste streams, effluents, manufacturing runoffs, sewage streams, air flow, and air and water extracts of environmental samples.

Background Art

It has been widely recognized that toxic substances, or toxicants, of natural and industrial origins can, even in trace amounts, cause morbidity and mortality in humans, domesticated animals, and fish and other wildlife. Toxicants can be present in trace amounts in air, soil, water, or foods and foodstuffs and can produce acute and chronic adverse somatic effects in humans or animals exposed to or ingesting these materials, as well as mutagenic, teratogenic or carcinogenic effects. Consequently, substances essential to life, such as surface and ground water sources of drinking water, must be tested for an array of toxic substances such as heavy metals, chlorinated pesticides, and volatile organic industrial chemicals. Many of these toxicants are present only in trace amounts and are very difficult to detect. Yet even in trace amounts these toxicants may harm exposed humans and animals. Similar concerns also apply to the protection of fish and wildlife which live in or near lakes and streams. Environmental toxicants, even at very low levels in aquatic water systems, are concentrated by biological activity. For example, this occurs with some chlorinated pesticides and polychlorinated biphenyls and other similar compounds,

causing deleterious effects on higher life forms, including both wildlife and humans who consume fish from contaminated waters.

Assays to detect the presence of toxic substances in environmental samples at very low levels and within a reasonable period of time are needed. Ideally, such assays are qualitative as well as quantitative, by indicating the type of chemical substance detected by a positive result. However, at present both qualitative tests to identify an unknown toxicant and accurate quantitative chemical analyses are very slow and expensive processes. Environmental mixtures present special difficulties because one sample may need to be divided into small aliquots to quantitate multiple constituents. Sometimes appropriate analytical methodology and toxicological information is not available to quantitate minute but toxic amounts of individual compounds, particularly new compounds.

The analyses are further complicated when multiple toxicants are present in environmental samples. Appropriately sensitive quantitative analysis may be impossible because of interfering chemicals. The interaction between the toxicants can result in additive, synergistic or antagonistic interaction with results that are difficult to predict. Nevertheless, even if quantitative analyses are impractical, sensitive qualitative analysis of samples to screen for the presence of deleterious, even if unknown, substances is of great use in evaluating the safety of the environment.

To provide effective tools in risk assessment and to cope with the problems of rapid assessment of the toxicity of environmental resources, scientists and regulatory officials have developed bioassays for quick screening or examining environmental samples which can be both economical and sensitive. Bioassays typically measure the response of biological preparations or whole

organisms to a sample of unknown constituents but do not identify or quantify the chemicals concerned, except for their biological activity. Ideally data from *in vitro* bioassay tests should correlate well with *in vivo* effects on laboratory animals and organisms in the environment and with conventional toxicological or epidemiological data. Some bioassays are based on relatively simple biochemical tests, such as single enzymes or groups of enzymes. Another type of bioassay is based on the responses of cells, microorganisms or whole organisms. Whole organism approaches are exemplified by the well known Ames test for mutagenicity which tests the effect of a sample on the mutation rate of a bacterial strain with a known mutation rate and by the Microtox test marketed by Beckman Instruments which assesses the quenching of bioluminescence from *Photobacterium phosphoreum* as a measure of a substance's toxicity.

Whole animal toxicity tests are frequently used to investigate the effects of environmental contaminants on aquatic species (Marking and Kimerle, 1979; Buikema et al., 1980), but such tests are limited by the number of animals which can be economically and conveniently studied, by problems in obtaining organisms of known background and parentage as well as by the difficulties of extrapolating from one species to another. Alternatively, *in vitro* tests are frequently more sensitive to the basic effects of a chemical and respond at concentrations at least as low as those affecting the whole organism (Ekwall, B., Ann. New York Acad. Sci. (1983) pp. 64-77). Consequently, appropriate *in vitro* systems should help separate the various interacting physical and chemical processes associated with an organism's reaction to injury and enable study of specific actions at the macromolecular level, without the complicating effects of multiple organ systems (Dipple, A., et al., Ann. New York Acad. Sci. (1983) pp. 27-33).

None of the foregoing tests, however, provides a toxicant profile. All of these tests are designed to measure levels of known toxicants, or to discern the level of toxicity present in the sample in general. Clearly there is a need for an efficient way to generate data which provide information on the levels of an unknown toxicant, a multiplicity of toxicants or an unknown type or multiplicity of types of toxicants in a sample. The present invention provides such an assay.

Disclosure of the Invention

The invention is directed to a method to obtain a qualitative and quantitative toxicant profile of a fluid which method comprises a) contacting said fluid with an assembly of a multiplicity of compositions, each of which compositions reacts with at least one toxicant so as to produce a detectable signal, and wherein said compositions are chosen so that each potential toxicant will react with at least one of said compositions, but no more than one potential toxicant reacts with all of said compositions; b) detecting each of said detectable signals; and c) evaluating said multiplicity of signals to obtain said profile.

In one embodiment a kit comprising at least one film and a plurality of biotargets is provided, the fluid is exposed to the biotargets and at least one change is detected.

In another embodiment, the biotargets and a reagent are combined in a separation device such as a centrifuge tube, to which is added the fluid; toxicants react with the biotargets and produce probes; the fluid is separated and the device is observed for separated probes.

Another embodiment employs a kit with a plurality of biotargets, a reagent and a 24-well plate. Standard amounts of biotargets and reagent are placed in each

well; fluid is added to at least some of the wells and the wells observed for changes.

Another embodiment employs a kit with a series of bioassay plates, each of which comprises at least one film, at least one type of biotarget and a housing to hold the film and biotargets.

Still another embodiment employs a cartridge comprising a series of bioassay plates, each of which has at least one film and at least one type of biotarget, with the biotarget being capable of a change when said biotarget is exposed to a toxicant. The cartridge is placed so that the plates contact the waste stream. Each plate generates a signal when its biotargets contact toxicants, so that the cartridge generates a profile. The profile is used to identify the toxicant or toxicants or type of toxicant.

Another embodiment employs a biomembrane which is attacked by at least one toxicant; a solution on a first and a second side of the biomembrane such that the first-side solution has a high concentration of electrolytes and the second-side solution has a low concentration of electrolytes; and a means for measuring the potential across the solution on the second side of the membrane. The test fluid is added to the solution on the first side of the biomembrane and the electrical potential across the solution on the second side is measured. The change in electrical potential from that of a low electrolyte solution indicates a toxicant in the fluid.

The profiles obtained are useful in identifying a single unknown toxicant as well as a multiplicity of toxicants. The plurality of biotargets capable of generating different changes permits profiling the toxicant by the change or changes detected, whereby the toxicant is identified by class or as a particular toxicant.

Brief Description of the Drawings

Figures 1A through 1D are schematic representations of a red blood cell (RBC) (A), a cutaway view of an RBC (B), a liposome (C) and a layered biotarget (D).

Figure 2A is a schematic representation of a cross-section of a biomembrane, such as an RBC membrane. Figure 2A is a schematic representation of a bioequivalent system.

Figures 4 through 10 are schematic representations of a series of bioassay plates as well as various cartridges for holding the plates.

Figure 11 is a schematic representation of a pump to move fluid through the cartridge which contains bioassay plates.

Figure 12 shows a schematic representation of a cross-section of a vial or well containing an assay plate with biotargets.

Figure 13 is a schematic representation of a cross-section of a vial containing several assay plates.

Figures 14A and B are schematic representations of assay paddles which are coated with biotarget(s), biocoatings and optionally film outer coats.

Figures 15A and B schematically show the effect of a surfactant on a biomembrane separating high- and low-chloride solutions.

Figure 16 schematically shows a biocoating (top) after it has been in contact with a toxicant (bottom) which breaks up the protein coating.

Figure 17 is a schematic representation of an effluent alarm system, which can be used to reroute contaminated sewage or levy fines on polluters.

Modes of Carrying Out the Invention

A. Definitions

By the term "fluid" is meant a liquid or gas. A "fluid potentially containing toxicants", also referred

to as a "test fluid" is a fluid to be tested which may contain toxicants. Examples of such test fluids are waste streams from chemical plants, paper processing facilities and other types of manufacturing plants, sewage streams, remediation sites, streams, lakes and oceans. Examples of test gases include airflows in manufacturing plants and other environments. Solid samples, such as soil samples, also can be used in the present invention, provided they are first added to a fluid, such as water. In addition, solid sample extracts can be prepared from such materials as solids, slushes, refinery streams, dump sites, and plant building materials.

By "toxicant" is meant a substance whose toxicity is known to be of concern, because it can damage organisms when present at anticipated levels.

By "film" is meant a thin, porous material through which toxicants and water can pass but not target receptors.

By "biotarget" is meant a composition which reacts with at least one toxicant. The biotarget may itself have one or more probes or indicator components. When the biotarget reacts with a toxicant, the biotarget indicates an observable change or affects a probe. Figure 1 shows several examples of biotargets.

One preferred example of a biotarget is the RBC (Figures 1A and 1B) which normally contains the probe hemoglobin. The RBC can be stabilized by well known techniques, such as glutaraldehyde treatment. Other examples of biotargets are liposomes, exemplified by Figure 1C. Figure 1D shows a multilayered biotarget with each layer containing a different probe; these containing a multiplicity of compositions that respond to toxicants. On a molecular basis, suitable biotargets include but are not limited to lipids, enzymes, and transport and

structural proteins, which react with at least one toxicant.

Other examples of biotargets are membranes with proteins (See Figure 2A), membranes without proteins, vesicles (such as indicator-filled liposomes or particles formed from various cell membranes), biocoatings (such as mucus, glycoproteins, surfactant coatings, fish dermis, and fish mucus), biomatrices (such as dermal substrate, collagen, keratin, extracellular and intracellular proteins such as RBC skeletal proteins) with and without biocoatings, cells from relevant structures (such as the lungs, mucous membranes, and gills), tissue or organ equivalents used alone or with multiple probes (such as an epithelial equivalent which comprises an underlying biomatrix to which epithelial cells are affixed and an optional biocoating). Another biotarget system (Figure 2B) utilizes a natural or synthetic biomatrix 46 in combination with a cell layer 47 and a biocoating 48, such as a mucous layer and/or a surfactant coating.

A probe is associated with the biotarget and changes in reaction to a toxicant. The change can be primary, secondary or even tertiary, in that the change is directly or indirectly caused by a reaction with the toxicant. A probe can be a fluorophore, a chromophore a chemiluminescing molecule, a naturally occurring region or group on a protein, or a lipid or an enzyme which can be detected or observed. Probes also can include ions and their transport molecules. Probes can include molecules naturally present in the biotarget. Probes also include enzyme activities, measurements of cell viability, and changes in tissue equivalents or models.

Also included among probes are protein components and glycoproteins, structural and enzyme proteins, as well as active, transport components. These probes can be part of the biomembrane, biomatrix, or biocoating systems. Transport proteins and enzymes allow passage

of components from one side to the other side of membranes and can serve as probes.

Also included among probes are receptor molecules on membrane or biomatrix surfaces. Actual key biotargets and specific toxicants can change activity structure or function.

By "reagent" is meant a solution containing solutes which are compatible with the biotarget(s). In some embodiments, the reagent is optional. In other embodiments, the reagent may be formulated with the biotarget or probe. If the biotarget is an enzyme, the reagent also preferably contains the probe or substrate upon which the enzyme acts. In some embodiments, the reagent contains buffers.

By "detecting a change" is meant registering a change in the test system, such as the visible change in the color of the reagent when a probe is affected or released, as for example, when hemoglobin is released from RBC biotargets. Observing a change is exemplified by a visible color change, an audible alarm, a measurable change in electrical charge or current, light, temperature, as well as mechanical changes, such as a change in fluid flow rate or spring action, and a variety of other changes which are discussed below. Depending on the type of change, the change can be observed by the human eye (color matching) and/or with a variety of equipment, such as a spectrophotometer, radiation counter, fluorimeter, and nephelometer. The observation can be made a one time ("a static change") or at a series of times ("a kinetic change").

By "waste stream" is meant any fluid discharge which potentially contains toxicants, for example, effluents from chemical plants and municipal sewage plants. A waste stream can also be inside a facility, such as fluid from an outlet of an individual chemical processing vat.

By "generating a signal" is meant giving rise to light or an impulse (such as voltage, current or magnetic strength) by which messages or information can be transmitted. When a signal is generated, it can sound an alarm or cause printing or an electronic read-out.

By "system" is meant a detection system which acts in concert with biotargets to detect toxicants and quantitate changes. The system typically comprises multiple biotargets and probes which provide a multiplicity of changes which in turn are used to profile the test fluid in a sophisticated manner. Such a system also can mimic an *in vivo* system, such as the fish gill test.

B. General Description

The invention relates to bioassay methods of quantitating and identifying the biotoxicity of fluids which potentially contain toxicants. Such test fluids include water and air. If a toxicant is in a solid form, the toxicant can be added to a fluid before assay by this method. The present invention provides methods to test aquatic toxicity and pneumotoxicity.

One preferred embodiment includes RBCs as biotargets. When the RBC membrane is disrupted or altered, hemoglobin, a red pigment, is released and is easily detected and quantitated visually or spectrophotometrically and the RBC becomes a colorless, shapeless ghost. Released hemoglobin can also react with some toxicants and change color, which also can be detected, particularly spectrophometrically.

In addition, the RBC membrane naturally contains a chemiluminescent molecule which acts as a probe. In the presence of a toxicant, the RBC membrane is altered and chemiluminescence changed.

The fluidity of the RBC membrane can also be studied and evaluated.

Other biomembranes include vesicles fabricated from portions of intracellular and cell membranes. For example, intracellular membranes (e.g., mitochondrial membrane) can be homogenized and assume the shape of small vesicles.

An example of a synthetic membrane is the liposome shown schematically in Figure 1C. Liposomes are stable, water-filled spheres consisting of a lipid bilayer and other optional components. Lipids form liposomes by aligning themselves in parallel: The lipids' hydrophilic ends touching the surrounding water and their hydrophobic ends facing each other (similar to the configuration shown in Figure 2A for biomembranes, where the hydrophilic ends (25) align at the number surface and the hydrophobic portions are tucked inside. Liposomes can envelop diverse molecules, probes or other chemicals.

Liposomes are constructed from many different types of lipids. The lipids in turn can be combined with other molecules, including but not limited to proteins, chemical probes and other chemicals. Liposomes can be stabilized by cholesterol, for example, to withstand certain toxicants but dissolve in the presence of other toxicant.

Figure 2A shows a cutaway of the biomembrane of a biotarget such as an RBC. This shows that the membrane is predominantly a bilayer of lipid molecules, whose hydrophilic or polar ends 25 line the surfaces of the membrane and whose hydrophobic ends 30 are buried inside the biomembrane. The polar groups are exposed to the outer water environment and to any toxicant. The toxicant causes at least one change in the membrane components, such as binding characteristics, UV spectrum and other characteristics depending on the toxicant's presence, binding or alteration of the membrane.

In contrast to the simple lipid bilayer of the liposome which usually includes the lipid structures 25

and 30 of Figure 2A. The RBC bilayer also has a fluorescent probe 35 imbedded in the lipid bilayer. When the bilayer configuration changes this probe 35 fluoresces. A protein chromophore 40 is an integral membrane protein which changes color with changes in the bilayer or when protein itself is perturbed. A surface protein enzyme 45 will have differing activity, depending on the protein lipid interactions and on the enzymes interaction with toxicants. The activity of enzyme 45 can be used to gauge the presence of toxicants. Also, interaction of the toxicant with the RBC membrane produces a change in its natural chemiluminescence.

The biotargets are arranged in a wide variety of arrays, including one or a series of layers, in a reagent, on a film. The arrangement of the biotargets in one or more layers permits the test fluid to interact in a uniform fashion with the biotargets.

The biotarget layer can be produced in a variety of ways, for example, by prefabrication or by centrifuging a test mixture as described below. The biotargets can be deposited on a film to form a thin layer. Alternately, the biotargets can be solubilized in a solution, which can then be poured onto the film. In other embodiments, the biotargets themselves receive a biocoating, are encapsulated, dispersed in solids, liquids or semisolids in biocoatings or biomatrices.

The biotarget solutions and reagents are chosen to be compatible with the particular biotarget(s). For example, if the biotargets are RBCs, the solution or reagent has a physiologic osmolality and pH. Solutions compatible with RBCs include but are not limited to normal saline, serum albumin, 5% dextrose, plasma preparations, and other protein-containing solutions. Reagents and solutions that are compatible with liposomes vary with the content of the liposome. Reagents which are compatible with a number of liposome compositions

include but are not limited to water, saline, alcohol in water, etc.

The solution for depositing biotargets optionally contains a dispersant, such as a minor amount of a starch or gel. After water evaporation, the starch or gel holds the biotargets in place. Examples of a gel include but are not limited to starch, agar, keratin and collagen.

The biotarget optionally has an underlying biomatrix. In the RBC, the underlying biomatrix is "skeletal" protein and intracellular stroma. In alternate embodiments, the biomatrix consists of extracellular protein such as collagen and elastin.

The biotarget includes or reacts with one or more probes, which are released or changed when the toxicant affects the biotarget. One probe for the RBC is hemoglobin release. Other examples are hemoglobin denaturation, fluorescence of proteins in RBC, chemiluminescence and enzyme activity in RBC membranes.

A wide variety of probes can be placed in the liposomes. Such probes include but are not limited to colored substances, substances which change color, and fluorophores. Enzymes are useful probes, because upon release from liposomes, they react with a substrate and create visible change such as color. Examples of probes include fluorescein, cholinesterase, dehydrogenase, tetrazolium salts, methylene blue, lactate dehydrogenase, glucose-6-phosphate, ATP, cytochrome C, lipases, proteases or any mixtures of these probes.

Another biotarget consists of biocoating. The toxicant alters, denatures, coagulates or destroys this coating or layer. Coatings can be protein-based, protein and surfactant-based, surfactant-based, extracellular matrix-based, collagen, keratin, structural proteins isolated from dermis, epidermis, generated extracellular matrices from macromolecules. One model of a biocoating is fish gill mucus for which a synthetic model is

constructed of polymerized protein networks made up of gelatin, globulins, plant globulins, solutions and semisolutions or other glycoproteins.

In one embodiment (Figures 3A and 3B), the biotargets are contained in a housing 15 which also includes two layers of film 10 to form a biotarget plate 1, although only one layer of film may be necessary if a gel dispersant is used. The other surface can alternately be a support.

The film 10 is a thin, porous, semipermeable membrane. It is thin for easy diffusion of the test fluid; however, if the fluid is sufficiently porous, the film can be thicker. The porosity of the film is an important characteristic. The film must be sufficiently porous to let the fluid potentially containing toxicants pass through at the desired flow rate. The pore size and number are varied to achieve the desired flow rate for the fluid to which the bioassay plate is exposed. The film's pores must be smaller than the biotargets. Generally, the film is relatively nonreactive with the range of toxic substances to which the film is exposed. Alternatively, the film is part of the assay system, turning color or otherwise changing when exposed toxicant(s).

To hold the film 10 and biotargets 20 in place, a holder 15 may be placed around the perimeter of the film. The requirements of the holder are not stringent. However, the holder must be made of a relatively rigid material which will not interfere with the toxicity test. Many plastics are resistant to the toxic materials to be tested and are acceptable for this purpose. Examples of suitable materials include but are not limited to stainless steel, polyvinyl chloride, ABS plastic.

In one embodiment, the film, biotargets and housing are arranged in the shape of a flat spherical or rectangular bioassay plate, as shown in Figure 3A. The

bioassay plate 1 is a flattened cube with a film 10 edged by a holder 15. In the cutaway view in Figure 3B, the holder 15 is shown to actually surround the edges of the upper and lower films 10. The biotargets 20 are contained between the upper and lower films 10. Such film sandwiches can be made individually or the film sandwiches can be fabricated in a continuous roll from which individual biotarget sandwiches are cut and compressed into the holder.

Additional embodiments include various combinations of the bioassay plate of Figure 3. Figures 4A-C show that a series of the plates of different shapes can be assembled to have series fluid flow. Figures 5A, B and C show different shapes for the bioassay plates, and there are spaces between the plates to permit easy circulation of fluid around both surfaces.

Figures 6A and B show rectangular bioassay plates 1 assembled into a series (A) and inserted into a partial cartridge 50(B). Figures 7A and B show a series of spaced bioassay plates (A) inserted into a partial cartridge 50(B).

Figure 8A shows spaced bioassay plates 1 inserted into a carrier 55 with slots 60. In Figure 8B partial cartridge 50 has upper and lower tracks 65 to slide plates into the cartridge 50 on one side. This arrangement permits the selection of plates which can perform a number of different tests.

Figures 9A and B are schematics of cartridges 50 which indicate arrangements of the bioassay plates 1 and fluid flow around the bioassay plates. In Figure 9A, the bioassay plates are generally parallel to the fluid inflow and outflow direction and are spaced to permit maximal flow. In Figure 9B the bioassay plates are perpendicular to the fluid inflow and outflow and are supported by strips 70 which separate the bioassay plates

from the lateral surfaces of the cartridge 50; flow is more turbulent in this configuration.

Figures 10A and B show the same parallel and perpendicular arrangements of the bioassay plates but additionally illustrate tracks into which the bioassay plates are inserted. The tracks are formed by placing feet to the sides of each bioassay plate. Bioassay plates can be assembled into a cartridge, or other experimental design, which are used to perform multiple assays. This multifunctional cartridge replaces current *in vivo* bioassays such as live fish assays, in which the gills are removed and examined or tested for such parameters as biomembrane transport, cell viability, and enzyme activity.

In the cartridges exemplified in Figures 4-10, each plate can be the same or different, in that the plate includes a specific set of biotargets or other types of assay system that are the same or different from those in the other plates. Although only a few plates are shown in Figures 4-10, any number of plates can be used.

A single biotarget such as RBC may have several, e.g. 20 or more, different probes to provide a toxicity profile of the toxicant or toxicants. The combination of plates or assays can be varied for the type of test fluid to be assayed, that is, the expected toxicants. In one embodiment, heavy metals, such as chromate, copper, cadmium, selenium and zinc, are assayed by analyzing protein alterations. Organics, such as PCB and pesticides, are assayed by their effects on other compositions by testing membrane fluidity and other factors, while other compositions can assay surfactants or detergents. Each of the compositions, contained in one or more biotargets, can be optimized for a particular toxicant that is known to occur in the particular type of test fluid. For example, very specific antibody tests can be used to identify dioxin, specific receptors can be

used to identify hormones such as insulin and growth hormones.

Model Systems for Toxicant Detection

The fish gill appears to be the principal target organ for many toxicants. A number of classes of toxicants disrupt the barrier properties of the gill epithelium leading to epithelial cell death. Fish death usually results from a combination of ionoregulatory, osmoregulatory and respiratory dysfunction. Thus, the fish epidermis could be a suitable model to determine irritation by toxic compounds, to study the toxicological mechanisms of pollutants, and to assess toxicants.

In the field of pneumotoxicity, *in vivo* animal studies have been the test method of choice. These studies are conducted in regulated chambers with small swine or pigs whose lung tissue is similar to human tissue. The changes in lung enzymes are important to the evaluation of lung damage. The lung has a surfactant biocoating, and this must be disrupted and the epithelial cells or cell membranes damaged to affect the level of enzyme activity in the epithelial cells. These methods have been used to evaluate parts per million (PPM) and parts per billion (PPB) of toxicants of chemical warfare agents, biological warfare agents, cigarette smoke, drugs for inhalation therapy, asbestos and resin damage, coal residues and other well known industrial hazards to lung tissue. An inexpensive, rapid *in vitro* model to mimic lung tissue in a reproducible manner is thus also suitable for toxicant determination.

The compositions for use in the method of the invention may be chosen, for example, to assess:

1. Enzyme activity (e.g. electron transfer, aldolase, phosphatase, ATPase, etc.);
2. Changes to the proteins and lipids in membrane biocoatings;

3. Color, fluorescent or chemiluminescent changes in biomembranes, receptors, biotargets, biocoatings;
4. Cell growth or death; and
5. Tissue destruction.

These compositions are conveniently disposed on bioassay plates which can be arranged in a specific or nonspecific order as shown in Figs. 4-10. This combination of tests mimics the behavior of fish gill.

Lung tissue can be mimicked with a primary biomembrane equivalent or model, a series of biotargets from subcellular particles, including cellular and living tissue with or without biocoating. The series of compositions, also conveniently disposed on bioassay plates has tests for one or more of the following:

1. Structural integrity of cell membranes;
2. Enzyme activities;
3. Coating disruption of natural lung surfactants or synthetic surfactants;
4. Transport enzyme activity;
5. Fish gill epithelium equivalent;
6. Cell viability; and
7. Microcalorimetry.

Structural integrity of cell membranes can be measured by RBCs or color-filled liposomes, either of which release a color probe when their membrane structural integrity is damaged. Enzyme activity may employ a pulmonary or other enzyme which when activated by a toxicant causes a color or other observable change. Coating disruption of pulmonary surfaces employs the biotargets coated with pulmonary surfactant or a substitute, wherein disruption of the coating causes the biotargets to release a probe.

Transport enzymes are inactivated by toxicants to convert measurably less of its substrate. Cells from or

related to those found in fish gill epithelium or other epithelial structures are used directly. Cell viability can be tested with a variety of cells such as an array of cultured cells in a biomatrix as disclosed in U.S. Patent No. 4,963,489. Microcalorimetry can be linked to an electrical probe and/or by liquid crystal display. Alternately, key structures can be observed for change associated with temperature changes.

Additional useful biotargets include fibroblast cells grown from fish or lung or mixed tissue cells grown from lung or gill or mucosa. These are not transplants but are obtained from stable lines of cultures cells.

The bioequivalent of the fish gill, lung, mucosal epithelial or other epithelial equivalents can be used alone or in conjunction with the other biotargets. Such biotargets can be in discs of film, enclosed, encapsulated, coating the film, or on extracellular matrices. These bioequivalent cultures can be attached to plates or placed in cuvettes, discs and films.

Figure 11 shows another embodiment of the invention. The chamber is filled with a fluid such as air, which the pump 140 moves into the cartridge 50 which contains multiple plates 1 with a diversity of biotargets as described above. After air circulates through the cartridge, it recirculates through a pipe 145 into the air chamber 140. The air need not be recirculated but optionally can be recirculated with low level toxicants to predict the toxicants' cumulative irritancy, acute irritancy and other long- and short-term effects.

In Figure 12 is shown the bioassay plate with 2 layers of film 10 holding biotargets 20, mounted in a vial or well. Optionally one layer of film 10 can be used if the biotargets 20 are held in place by a gel. Figure 12 also depicts the general appearance of a well in a 24- (or other convenient number) well plate. In practice the test fluid is applied to the top film layer

and is permitted to diffuse into the biotarget area. There, the toxicant reacts with the biotargets which in turn act on probes which pass through the lower film and into the chamber 147 below the film where the change(s) is detected.

In Figure 13, there are several bioassay plates which can be used to determine the same or different toxicities. If the plates are the same, occurrence of a change in chamber 147 is timed and happens quicker with more toxic substances. If the bioassay plates are different, multiple probes can be detected in chamber 147 by various methods (e.g., spectrophotometric analyses at different wavelengths).

In Figure 14 the biotargets are mounted on paddles 150. One end of a long narrow strip 155, for example, of wood or cardboard is the handle 155, while the other end of the strip is coated with at least one type of biotarget 160. The coated end of the paddle can have more than one type of biotarget. An example of multiple biotargets is shown in Figure 14B. Three types of biotargets 165, 170, 175 are on the top surface of the strip, while the opposing surface has an additional type of biotarget 160. The biotargets are coated with a biocoating 180, such as described above. In addition, the biocoating can be covered by the type of film 185 described above.

This paddle is adapted to field use, for dipping in streams and ponds. It is removed from the fluid and can be immediately read, or the paddle can be rinsed off before the biotargets are observed for a change.

Examples

The following examples are intended to illustrate but not limit the invention.

Method for Synthesizing Liposomes

Pipette phosphatidylcholine containing 2% phosphatidic acid in organic solvent into a thick-walled tube or vial. Allow the solvent to evaporate while rotating the tube to spread the lipid in a film over the tubes inner surface.

Add 0.25 M sucrose, 0.01 M Tris buffer, 0.001 M EDTA, pH 7.4, and sonicate the mixture using an M.S.E. sonicator at setting 5 for 10 min. The tube is kept cool by immersion in ice during the sonication.

Centrifuge the sonicated mixture for 90 min at 105,000 g to pellet any undispersed lipid and remove the upper three quarters of the supernatant preparation.

Sonicate the suspension until clarified using a bath sonicator; this may require up to several hours, and should be done under nitrogen or argon. For a more complete description of liposome techniques, see Deamer and Uster (1983) In Liposomes. Ostro, M.J. (ed), Marcel Dekker, Inc., New York, p. 27.

Incubate the probe with the liposomes for 1-5 hr. When the probe is a protein, a surfactant such as 0.1% (w/v) Lubrol PX (polidocanol, available from ICI) may need to be added. Collect the liposomes by centrifugation at 300,000 g for 30 min.

An alternate method or preparing liposomes is the freeze-thawing technique, which uses detergents. Detergent-free protein is combined with an aqueous dispersion of lipids. The mixture is sonicated or quick-frozen in liquid nitrogen, thawed and sonicated.

Assay for Cell Viability

Fibroblast cells from fish gills or other sources are tested for viability and enzyme activity after exposure to toxicants.

Gill tissue is removed from the fish and sterilized in a solution containing antibiotics. This tissue then

is minced under sterile conditions and kept in an appropriate cell culture medium. Next the tissue is centrifuged and supernatant is discarded. The pellet is resuspended in trypsinization medium in a flask with a magnetic stirrer. The tissue is allowed to sediment by gravity. The supernatant containing dissociated cells is drawn off, centrifuged and resuspended in proper medium for further propagation. Derived cells are kept in a CO_2 incubator at appropriate temperature and CO_2 concentration. Alternately, cultured or isolated fibroblasts can be cryopreserved. At a convenient time, the cells are thawed and mounted on bioassay plates between two layers of film (or within an agar or biomatrix coating) and used to test for toxicants. Alternately, as shown in Figure 2B, the cells can be grown up on a biomatrix 46 such as agar or an extracellular matrix. Optionally, the cells have a biocoating 48, such as a mucus-like substance. Depending on the reagent used, any of the following parameters of cell viability and activity can be determined: cell growth, neutral red uptake (NRU), neutral red release (NRR), enzyme activity (e.g., ATPase activity, aldolase activity), gene stress and cell growth numbers.

Fish Gill Epithelial Tissue Equivalent

Similar to above, and as shown in Figure 2B, a living epithelium equivalent or model is prepared by culturing fish cells 47, such as fibroblasts, mucous cells, or epithelial cells alone or with other stromal cells on a biomatrix 46 scaffolding material, such as fish gill dermis substrate, or other dermal substrate, such as collagen or keratin. Optionally the epithelial equivalent can be covered by a biocoating 48 or extracellular matrix or coating. Such tissue cultures are mounted in bioassay plates between two films or on a biomatrix.

Tests for Surfactant

Figure 13 shows a schematic for before (A) and after (B) surfactant exposure. Figure 13A shows a container 190 (which can be as small as a cuvette. The container has electrodes 195 on either side which are wired to a meter 200. Below the bilipid membrane 205 is placed a solution with a low concentration of chloride ions 210. On top of the membrane is placed a solution with a higher concentration of chloride ions 215.

Figure 13B shows that the bilipid membrane 190 has been sufficiently destroyed by surfactant to let chloride ions diffuse from the high chloride solution 215. The meter now shows current running through solution 210. Even if the current difference before and after application of the test fluid had not been determined, a difference between a standard low-electrolyte solution and the post-toxicant electrolyte solution can be determined.

An electrical charge also can be created using differences in osmotic pressure across a matrix or membrane. Perturbation of the biomatrix or biomembrane can cause changes in resistance or be measured as ion flow across the membrane, or increased current flow. These embodiments can be integrated into circuitry for alarms to indicate the presence of toxicant in a test fluid.

Another assay measures the electrical potential across a wire that is initially insulated or coated with a substance, such as keratin, which is dissolved off the wire by a surfactant. When the keratin insulation dissolves, a change in the electrical potential across the wire is recorded.

Another embodiment is a wire embedded in the biomatrix, biomembrane, or biocoating. Upon destruction of the biotarget, the change in electrical potential is detected.

Another surfactant test utilizes a screen with a biocoating. As test fluid flows through the screen, toxicant dissolves the biocoating. Fluid flow through the screen increases, or the pressure drop across the screen decreases.

Assay for Heavy Metals

With heavy metals, the primary targets are proteins and particularly negatively charged proteins. Heavy metals will affect biotargets, such as proteins or enzyme components, and can cause cell death. Therefore, a change in protein transport, enzyme activity, hemoglobin denaturation, biomatrix and biocoating aggregation indicate the presence of heavy metals.

Figure 14 shows a biocoating at the top which is a regular matrix of tritiated protein. Upon alteration by heavy metal-containing fluid, the biocoating has the irregular appearance of the lower half of Figure 14. The protein is broken into short strands soluble in water. The fluid over the biocoating can be tested for tritiated proteins. Because the biocoating itself is altered and irregular, it will not reflect as much light as the untreated coating: Altered light absorbance also can be observed.

In addition, a cartridge to profile heavy metal contamination is assembled of the following types of biotargets:

Coating denaturation
Lipid fluidity
ATP inhibition
hemoglobin denaturation
cell death

Each bioassay plate is read for a change. The combination of presence or absence of changes in the bioassay plates is a profile for the toxicant. If there is mercury in the water, the following are observed:

coating denaturation
no lipid fluidity change
ATP inhibition
hemoglobin denaturation
cell death

Assay for Biomembrane Transport

A membrane with a chlorine pump can be used to detect the effect of the test fluid on the pump. The chloride ion concentration can be read by gas chromatography (GC), high pressure liquid chromatography (HPLC) and spectrophotometer. The change in chloride gradient over time indicates deterioration of the biomembrane's chloride pump.

In this embodiment, the cartridge contains several plates with the same biotarget Cl- pump membrane. The effluent, or test fluid, contacts all plates in the cartridge. Individual plates are removed from the cartridge at predetermined times. Chloride ion concentration and gradient are determined. Malfunctioning chlorine transport determined by this method is the foremost cause of fish death and is called bluefish gill syndrome. The fish chloride pump also is affected by low levels of chronic exposure.

Assay for Enzyme Activity

Glucose-6-phosphatase (G-6-P) is an enzyme in the RBC membrane. When the RBC membranes are exposed to toxicants, the toxicant can react with G-6-P and decreased G-6-P activity can be detected. In the presence of nontoxicants, G-6-P activity is not changed.

Assay for Organics (PCB and Pesticides)

The cholinesterase enzyme is incorporated into lyposomes when they are prepared as discussed above. Cholinesterase is specifically inhibited with pesticides

which are chlorinated hydrocarbons. Inhibition of this enzyme can be detected as decreased production of the end-product or lack of a change in the concentration of the enzyme's substrate.

Assay for Detergents

Soaps, bile salts and saponins cause RBC hemolysis, as do surfactants. Moreover, the hemoglobin which is released by hemolysis precipitates, disperses and denatures in the presence of detergents. Putnam, "The Interactions of Proteins and Synthetic Detergents," In: Advances In Protein Chemistry, Vol. 4, 1948, pp. 79-122. An bioassay plate is constructed to test detergents. The bioassay plate has RBCs between two films which are sufficiently porous to let in detergents but not to let out hemoglobin.

The detergent-sensitive bioassay plate is exposed to a test fluid suspected of containing detergent. After a sufficient time, the bioassay plate is removed from the test solution and is observed for the presence of released red or denatured brown hemoglobin.

Chemiluminescent Bacterial Toxicity

Bioluminescent bacteria are sensitive to a variety of toxicants. Bioluminescent bacteria are available freeze-dried and are rehydrated when required for use. One such type of luminescent bacteria is Photobacterium phosphoreum B-NRRL 11177 (available from Microbics Corp., Carlsbad, CA) and which is discussed in Bulich et al., J. Bioluminesc. Chemiluminesc. (1990) 5:71-77.

In a similar manner to that described above for RBCs, freeze-dried bacteria can be placed between the two films of an bioassay plate of the present invention. The bacteria can be accompanied by other chemiluminescent-activated dyes as well as stabilizing agents, including gels. For testing a potentially contaminated fluid, the

plate is placed in the test fluid for a standard period of time. The time varies with the permeability/flow characteristics of the film.

The luminescence after a standard time is compared with the luminescence of a control bioassay plate which is not exposed to toxicants. The higher the luminescence of the bioassay plate exposed to potential toxicant, the lower is the toxicity of the test fluid.

Assay for Coating Disruption

This assay utilizes the bioassay plate format described above. The difference here is that between the two layers of film is a complex tissue culture raised from the esophagus of the New Zealand black flounder Rhombosolea retiaria. Shephard, Embryol. Zool. (1982) 55:23-34. In addition, microelectrodes sensitive to sodium, potassium and calcium cations are used to measure the gradients of ion activity within the mucous layer which lies on top of the tissue culture. The electrodes are connected to a recording apparatus, and their potential is measured relative to a grounded electrode. Signals from the electrodes can be amplified by a variety of high-impedance instruments, such as a Radiometer electrometer (PHM62) and an Analog Devices 311K. The very high impedance of the calcium-sensitive electrodes may be overcome by using an I.L. picometric amplifier (Model 181) with negative capacitance compensation. To assure that the microelectrodes measured only the mucous changes, not intracellular changes, the resistance of the microelectrode circuit is also monitored. An AC pulse is supplied from a stimulator to the amplifier input of the electrode, via a small air-gap condenser. This drives a capacity-coupled current through the resistance of the microelectrode circuit. The resulting potential fluctuation "seen" by the amplifiers is related to the magnitude of that resistance. If the microelectrode

penetrates the tissue surface, the resistance of the circuit is altered by the influence of the cell membrane and there are fluctuations of potential. Recordings are made on twin-channel pen recorders.

Before test measurements, the electrode circuits and tissue cultures are standardized and calibrated. Then the bioassay plates containing the electrode-tissue culture system are exposed to fluids potentially containing toxicants. The current changes are related to the intensity of the effect of the toxicant on the mucous layer.

Bacterial Toxicity Test

An bioassay plate is prepared from two layers of film, preferably cellophane, between which there are bacteria. The bacteria are selected for their susceptibility to a variety of toxicants, including benzene, nitrotoluene, chlorinated benzenes, glycerine, aldehydes, cresols, petroleum products such as kerosene and phenols. Suitable bacteria include pseudomonas, bacillus, aerobacter, azotobacter, arthrobacter, micrococcus and nocardia. The bacteria are provided in dry powdered form. In an alternate embodiment, N-fixing, N-cycle bacteria are used to test the aquatic toxicity of effluents in sewage treatment plants, industrial pond sites, and the fermentation of organic components in water.

The dried cultures are mixed with a dry, powdered gel which contains probe dyes which change color when the bacteria die. Suitable gels include but are not limited to agar, starch and gelatin. Suitable probe dyes include but are not limited to fluorescein, tetrazolium salts, methylene blue, or any mixtures of these dyes.

In use, the bioassay plate is placed in a sample of sewage or other waste water. The plate is removed after a sufficient time for toxicants to diffuse into the

bacteria-gel layer and cause a significant change, most desirably about 15 minutes to 3 hours, although the time interval can be varied, depending on the parameters of the film chosen.

Profiling Toxicants

A set of bioassay plates such as depicted in Figures 4 through 10 can be chosen to assay major toxicities of toxicants. By the selected effects, it can be determined which classes of toxicants are present. One set of assays for biotargets for profiling includes the following:

biomembrane integrity
protein conformation
membrane fluidity
transport activity
dehydrogenase activity
electron transport function

The pattern of results recorded on the various plates allows for distinguishing pesticide toxicity from heavy metal toxicity in the following manner:

TEST	PESTICIDE	HEAVY METAL
biomembrane integrity	yes	yes
protein conformation	no change	changed
membrane fluidity	yes	no
transport activity	no	yes
dehydrogenase activity	no change	changed
electron transport function	no	yes

Layered Configuration

In this example biomembranes are lined with at least one probe. The layered configuration generally follows Figure 2B. This is accomplished by 1) covering a biomatrix 46 with a layer of cells 47 and an overlying biocoating 48 such as mucus, or 2) selecting probe

molecules which are hydrophobic and capable of associating with a lipid bilayer. These liposomes contain the probe molecule(s). Then any exposed probe molecule(s) are stripped off the exterior, so that the test kit only offers liposomes with internal probe molecules. Other methods of coating probe molecules on lipid bilayers are known to those in the art.

In practice, cholinesterase is a suitable probe. When a pesticide toxicant destroys the bilayer and detaches cholinesterase, cholinesterase is released and free to react with its substrate.

This test also can be used to identify or help profile cationic heavy metal poisoning because the heavy metals also poison an enzyme such as horseradish peroxidase.

Paddle Configuration

As shown in Figures 14A and B a solid support, such as a stick or long narrow piece of cardboard, is coated with at least one type of biotarget 160, followed by application of a suitable biocoating 180, such as a lipid bilayer.

In Figure 14B, a solid support is coated with four biotargets, such as enzymes 165, hapten antigens 170, dye substrates 175 and fluorophores 160. Optionally, the biotarget molecules are covalently bound to the support, which will permit the support surface to be assayed by the appropriate technology. Then over the biotarget layer is applied a biocoating 180, such as a lipid bilayer.

The coated support is exposed to a test fluid for a standardized amount of time. The biocoating is destroyed. The change in the biotarget molecule can be visually observed by fluorescence, luminescence and/or chemiluminescence. Alternately, when the paddle is placed in a chamber with enzyme substrate, the exposed

biotarget molecules cause a reaction or response to the enzyme substrate or other suitable molecules. The amount of destruction of the biocoating is an indication of toxicant(s) level.

Lethal Toxicity Profile Test

In this embodiment, the profile test utilizes cartridge containing a series of bioassay plates, whose geometric configuration is not important (can be any of Figures 4 through 10). In one embodiment, the plates contain the following types of bioassays:

intestinal biocoating equivalent
biomembrane alteration model
mucosal cell equivalent
blood partition coefficient model
cellular toxicity model

Each bioassay plate is exposed to a range of concentrations of the test toxicant, new drug, new food additive, etc. The concentrations range from putatively nontoxic to highly toxic. Positive and negative controls of known safe and unsafe substances are tested in parallel with the unknowns.

The scores with the various profiles can be used to develop a LD_{50}, which is correlated with *in vivo* toxicity. This also highlights which organ systems might be expected to have the most damage. The blood partition coefficient demonstrates the affinity of the toxicant or drug for aqueous and lipid compartments. The first site of transport for poisons is often across the intestinal mucosa and into the blood and then into target organs. If sufficient exchange occurs, the toxicant is removed from blood but is lipophilic and concentrates in the fat.

Biotoxicant Alarm System

The cartridge assays described above are adapted for use as pollution alarms. Bioassay plates are wired to a central location where changes in electrical current are recorded on strip charts. Excessive changes in current set off audible alarms. A central location receives the input and can dam or redirect the toxicant fluid or can levy fines based on the changes recorded at the central location.

One such system is shown in Figure 17, which shows a portion of the industrial area of a city's sewer system. Letters A through F represent the various facilities capable of passing toxicants into the system. Circled V's represent valves which can alter sewage flow. Numbered circles 220 through 235 indicate the locations of cartridges of wired bioassay plates which send signals to the city's primary sanitation plant. Sewage passes from D and F toward the plant. If F pollutes, the test cartridge 235 sends a signal to the plant. The valve adjacent to the reservoir closes and F's pollution goes into the reservoir which prevents damage to the primary sanitation plant. If A through E pollute, the valve adjacent to the primary plant can be closed to redirect the pollution. Cartridges 220-230 are located to help identify the source of the pollution. For example, if cartridge 225 is signalling, but not cartridge 220, B is polluting; if cartridge 230 signals, but not cartridge 225, A is polluting.

This invention has been detailed both by example and by direct description. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above. Those equivalents are to be included within the scope of this invention.

Claims

1. A method to obtain a qualitative and quantitative toxicant profile of a fluid which method comprises:

a) contacting said fluid with an assembly of a multiplicity of compositions, each of which compositions reacts with at least one toxicant so as to produce a detectable signal, and wherein said compositions are chosen so that each potential toxicant will react with at least one of said compositions, but no more than one potential toxicant reacts with all of said compositions;

b) detecting each of said detectable signals; and

c) evaluating said multiplicity of signals to obtain said profile.

2. The method of claim 1 wherein each of said compositions independently produces a detectable signal by reacting with at least one toxicant to produce a response which response is selected from the group consisting of alteration of biomembrane integrity, alteration of protein conformation, alteration of membrane fluidity, alteration of membrane transport activity and alteration of enzyme activity.

3. The method of claim 2 wherein each detectable signal is selected from the group consisting of emitted or absorbed light, emitted or absorbed heat, an electrical signal and an audible signal.

4. The method of claim 1 wherein at least one said composition is an enzyme; and/or

wherein at least one composition comprises a membrane.

5. The method of claim 4 wherein said membrane is a red blood cell and the response of alteration of membrane integrity is hemolysis; and/or

wherein said membrane comprises a coating, comprised of polymerized protein networks.

6. The method of claim 1 wherein said assembly is a synthetic model of fish gill or lung tissue.

7. The method of claim 1 wherein the fluid potentially containing toxicants is selected from the group consisting of stream water, sewage effluent, ocean water, air, soil run-off, an air stream, an air extract, and mixtures thereof.

8. The method of claim 1 which further comprises providing an alarm system responsive to predetermined profiles, whereby said predetermined profiles activate said alarm system.

9. The method of claim 1 wherein said contacting, detecting and evaluating are conducted in a continuous mode.

10. A method to monitor a fluid for the presence of one or more toxicants, which method comprises:

a) contacting said fluid with at least one composition which reacts with said toxicant so as to produce a detectable signal;

b) providing an alarm system responsive to said signal so as to activate said alarm system to produce an audible sound; and

c) detecting said audible sound.

11. A method of monitoring a waste stream for pollutants, said method comprising

a) providing a cartridge comprising a series of bioassay plates, each bioassay plate comprising

i) at least one film;

ii) at least one composition which reacts with at least one toxicant to generate a signal; and

iii) a housing to hold the film and the composition;

b) placing the cartridge so that the plates contact the waste stream;

c) detecting the signal generated by each plate so that the cartridge generates a profile; and

d) using the profile to identify the toxicant or class of toxicants.

12. A method for assaying a fluid potentially containing toxicants, said method comprising the steps of

a) providing a kit comprising

i) a biomembrane which is attacked by at least one toxicant;

ii) a solution on a first and a second side of the biomembrane, said solution on the first side comprising a high concentration of electrolytes and said solution on the second side comprising a low concentration of electrolytes; and

iii) a means for measuring the potential across the solution on the second side of the membrane;

b) adding the fluid to the solution on the first side of the biomembrane; and

c) determining the electrical potential across the solution on the second side of the biomembrane;

whereby a change in electrical potential from that of a low-electrolyte solution indicates a toxicant in the fluid.

13. A method for determining the aquatic toxicity of a fluid, said method comprising the steps of

a) providing a kit comprising a fish gill equivalent or model;

b) exposing the fluid to the fish gill equivalent or model; and

c) observing a change, whereby occurrence of the change is correlated with fish gill irritation and corrosion.

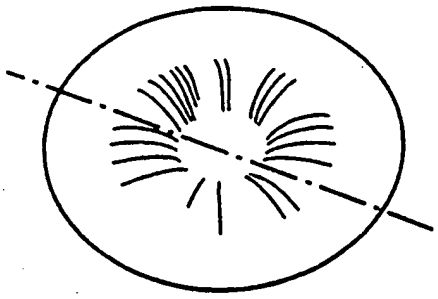


FIG. 1A

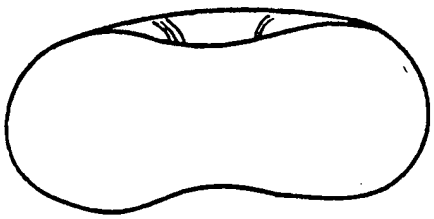


FIG. 1B

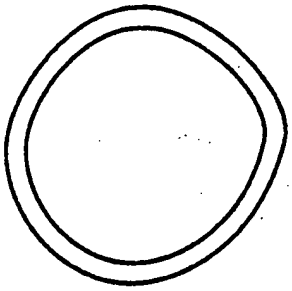


FIG. 1C

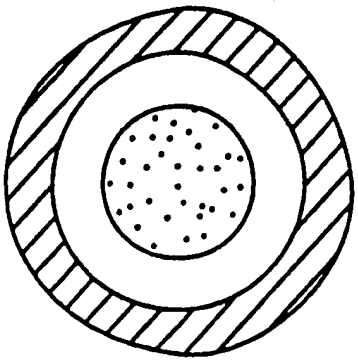


FIG. 1D

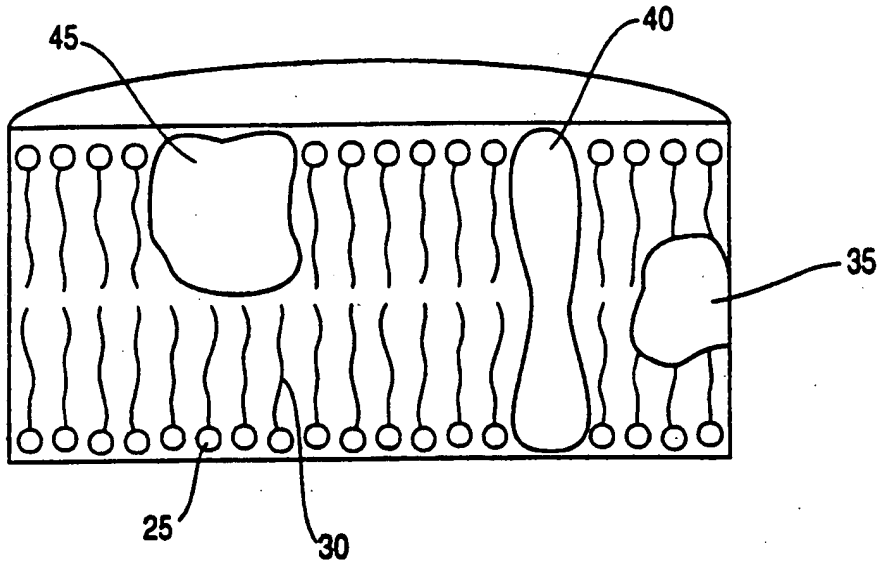


FIG. 2A

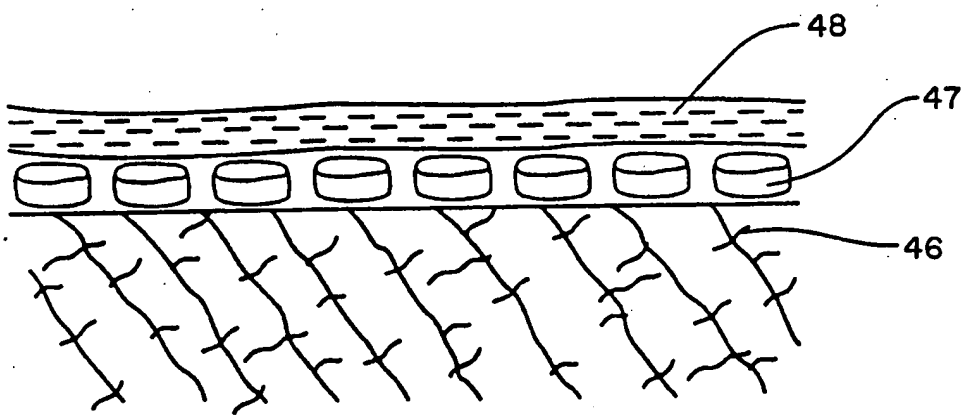


FIG. 2B

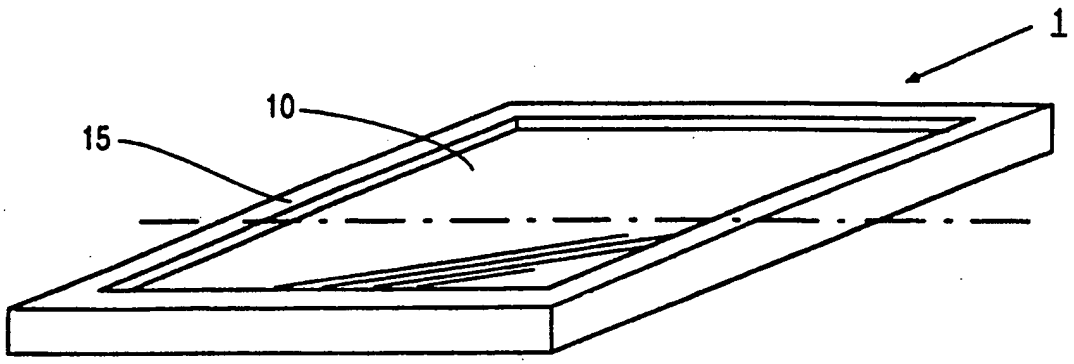


FIG. 3A

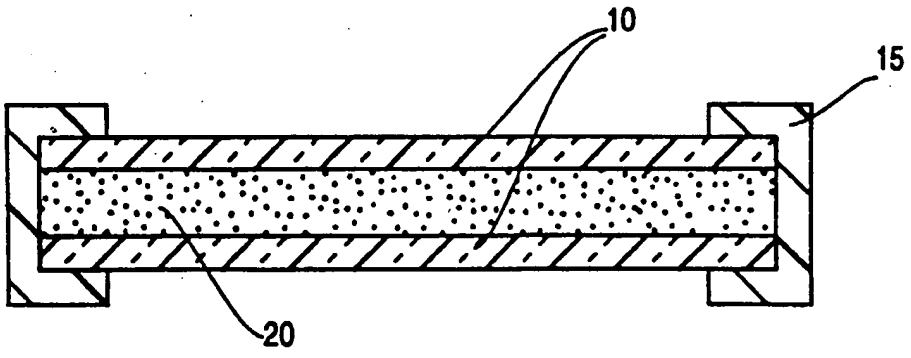


FIG. 3B

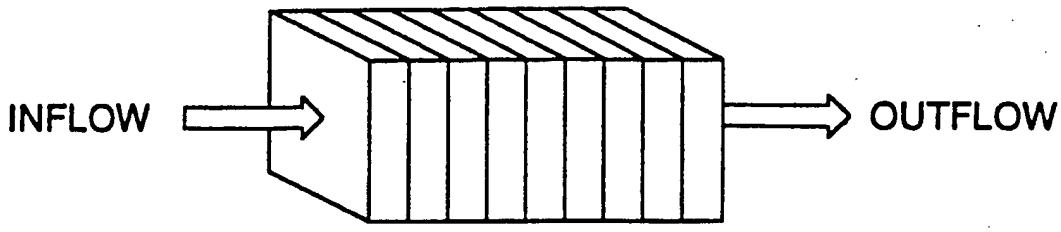


FIG. 4A

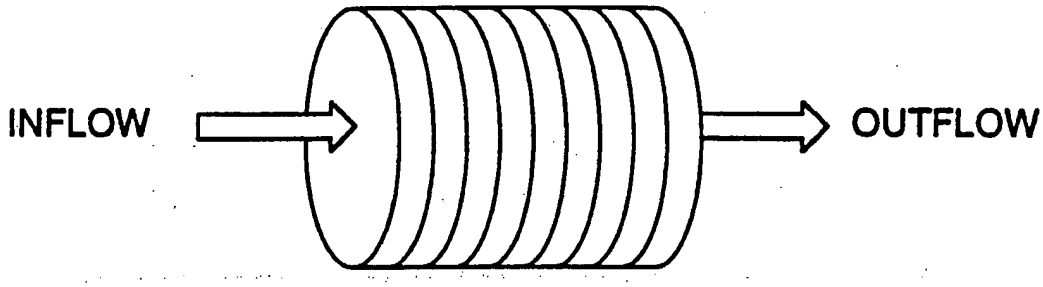


FIG. 4B

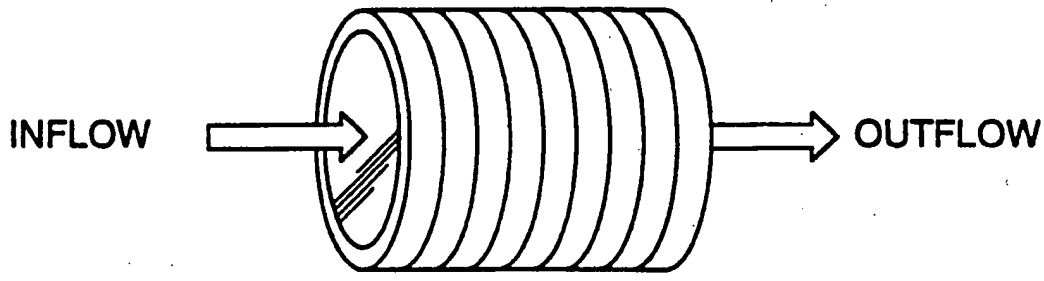


FIG. 4C

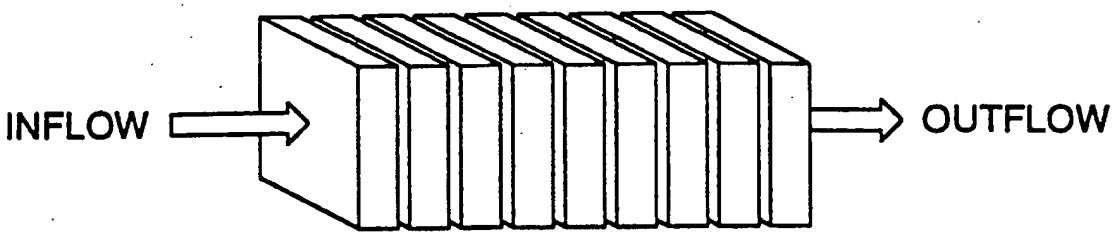


FIG. 5A

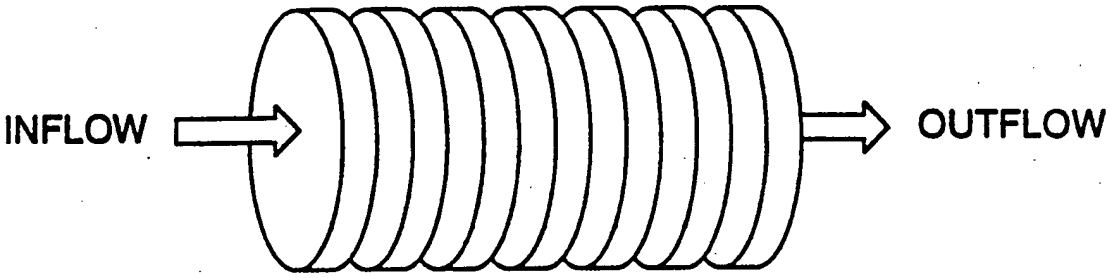


FIG. 5B

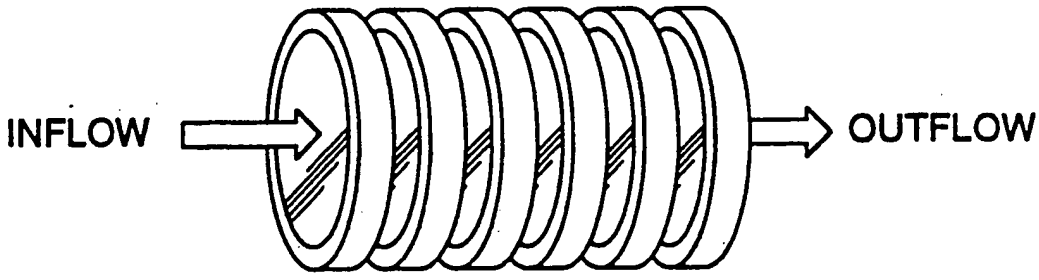


FIG. 5C

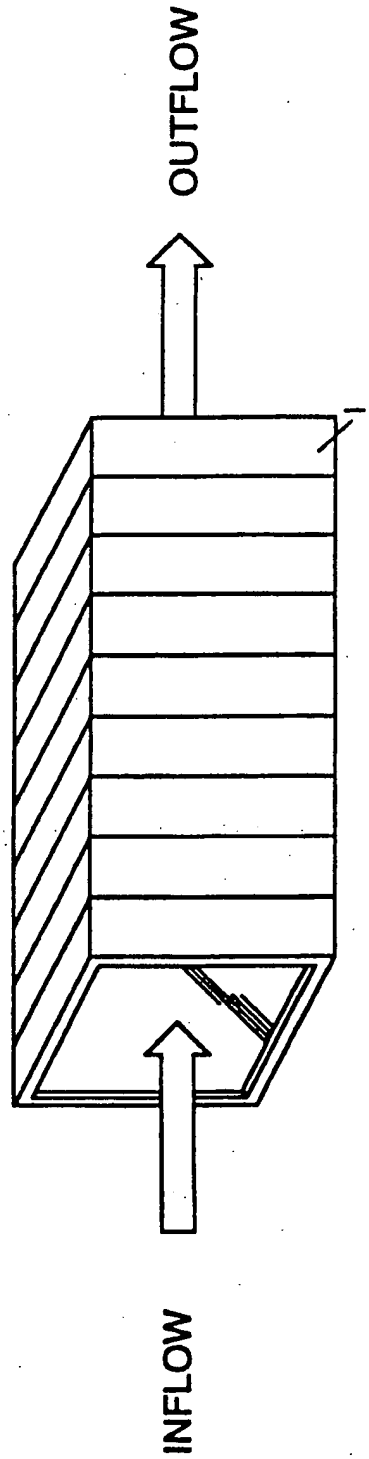


FIG. 6A

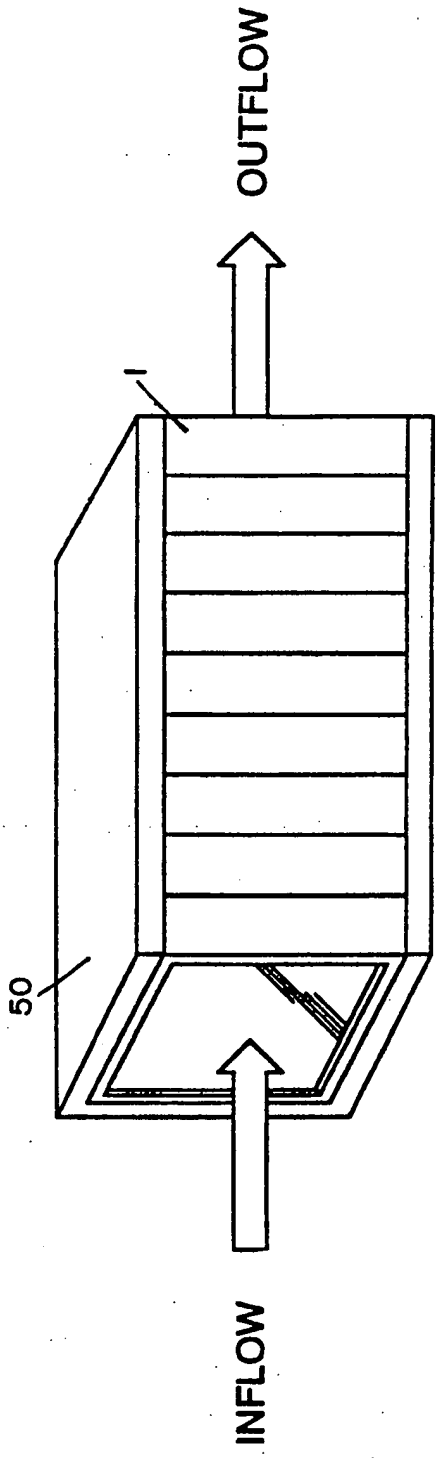


FIG. 6B

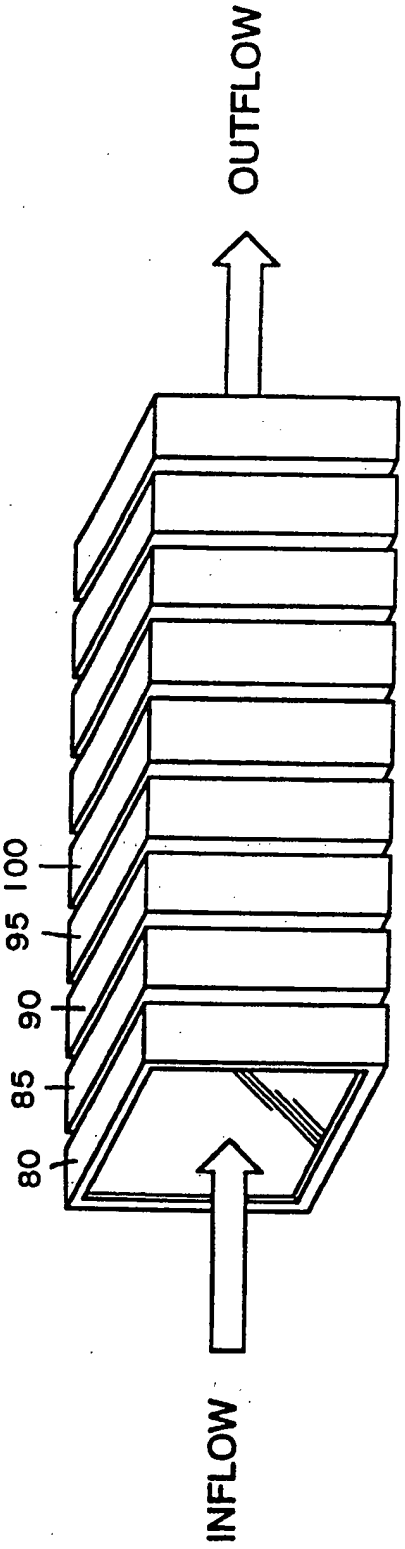


FIG. 7A

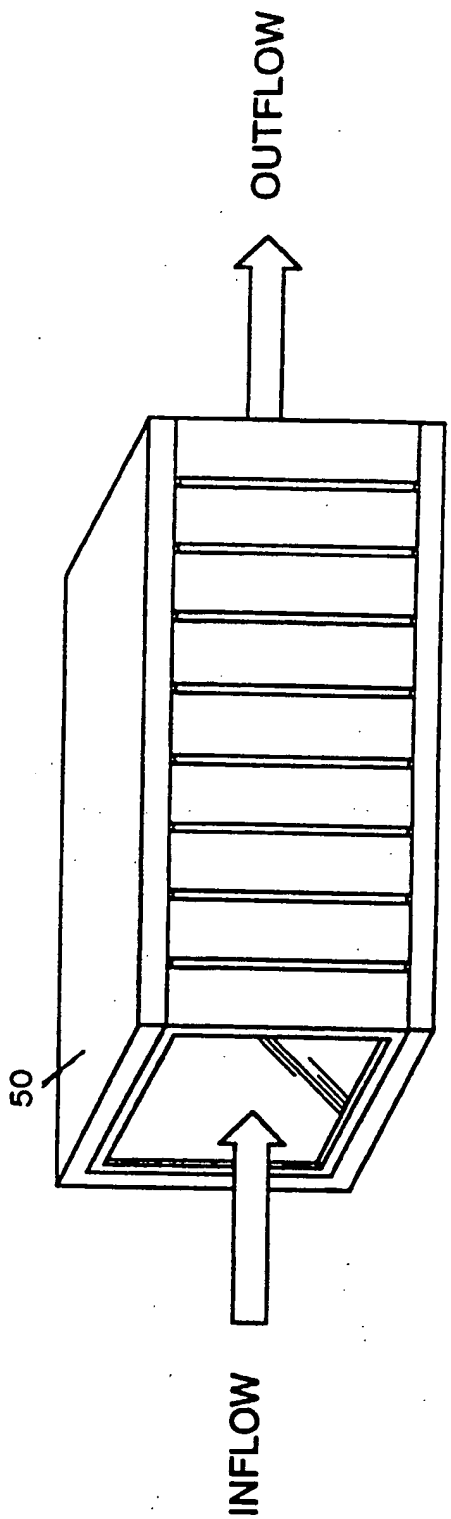


FIG. 7B

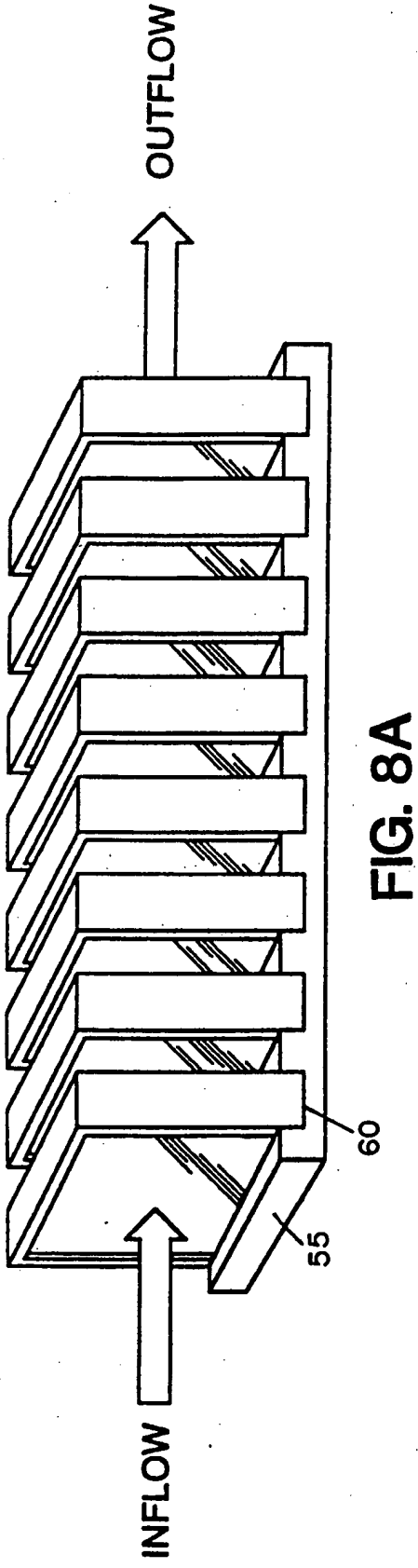

OUTFLOW
INFLOW
60
55

FIG. 8A

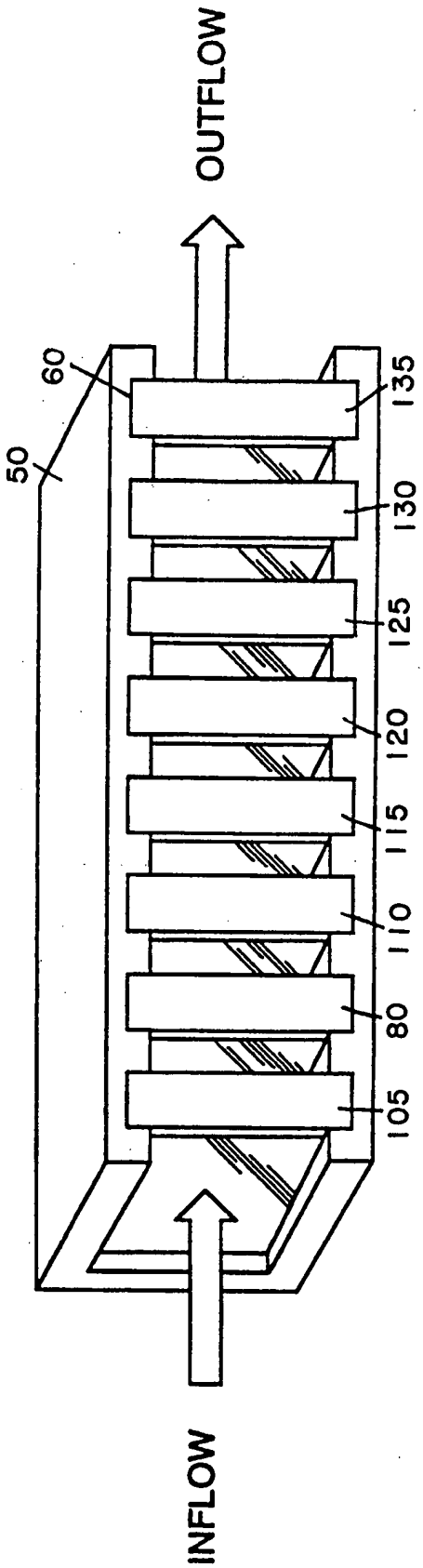

OUTFLOW
INFLOW
60
50
135
130
125
120
115
110
80
105

FIG. 8B

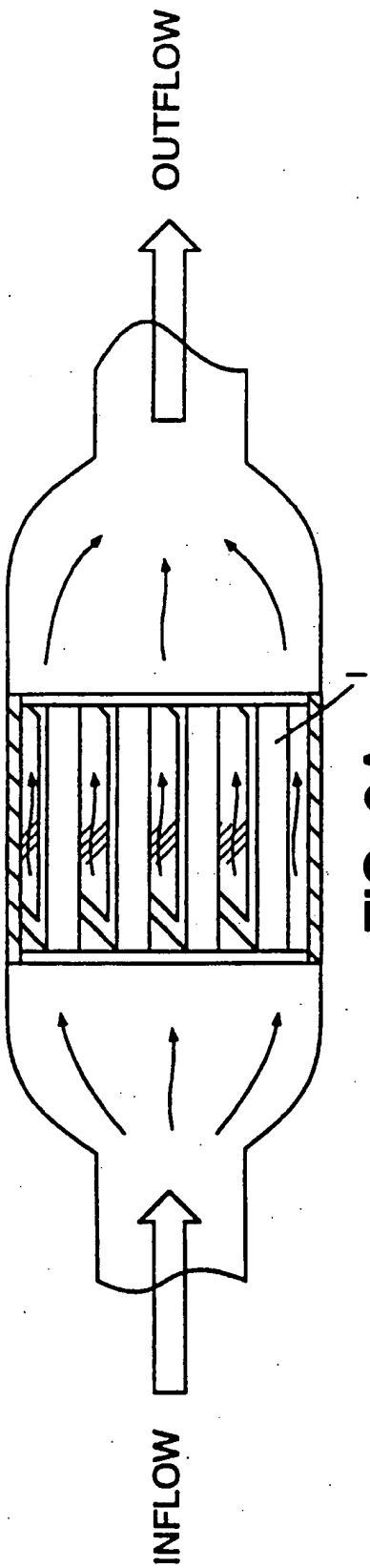


FIG. 9A

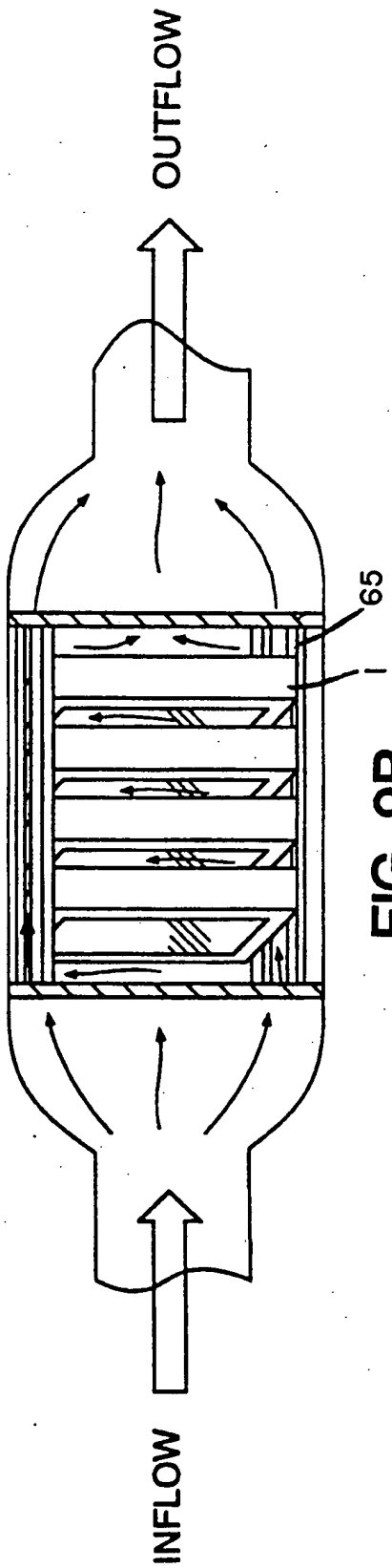


FIG. 9B

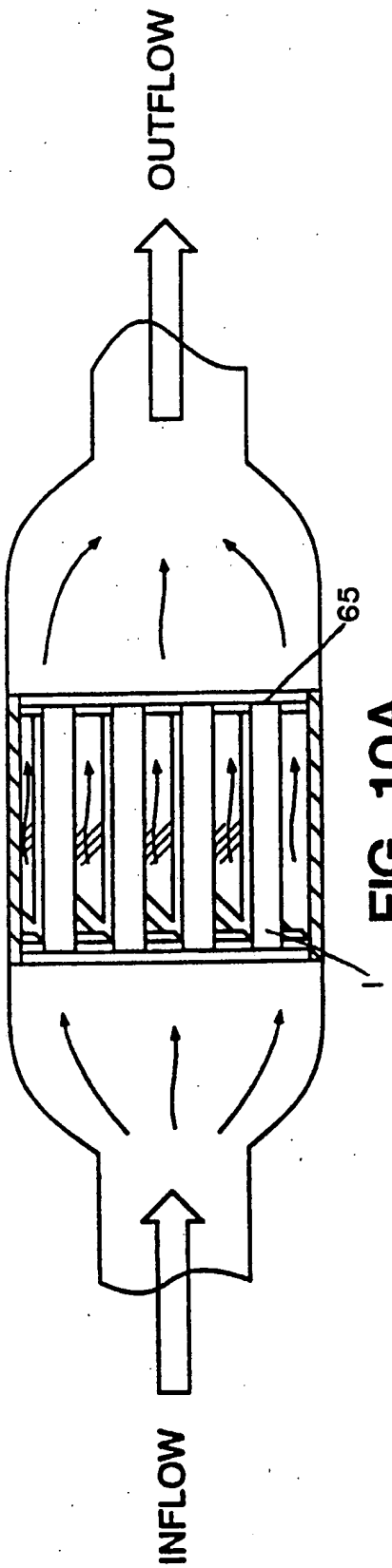


FIG. 10A

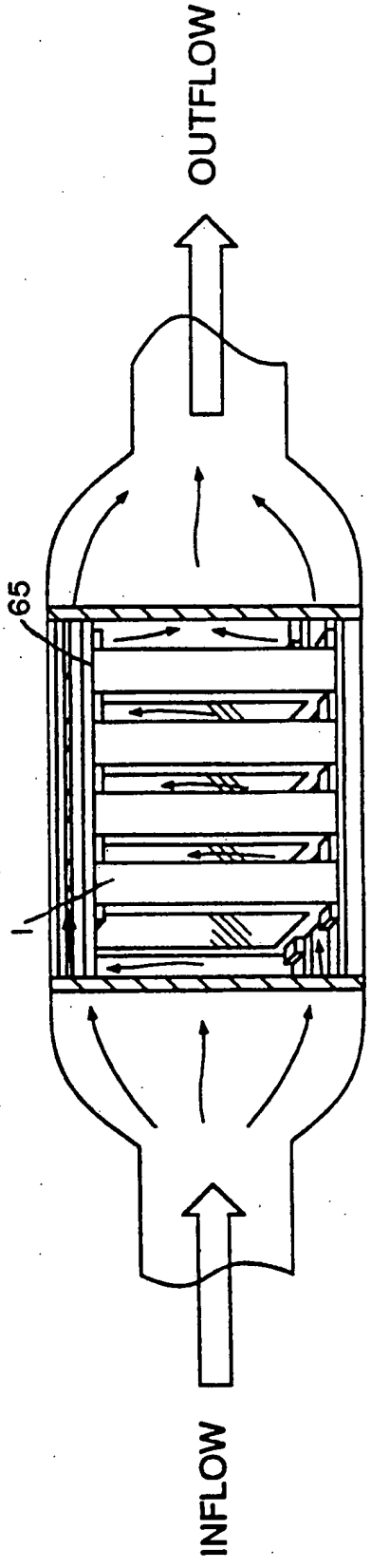


FIG. 10B

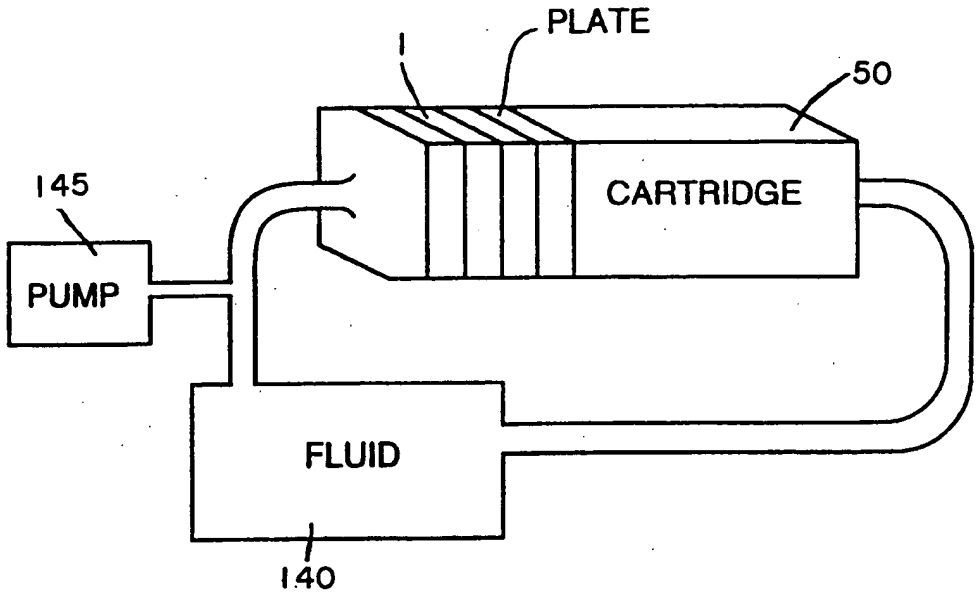


FIG. 11

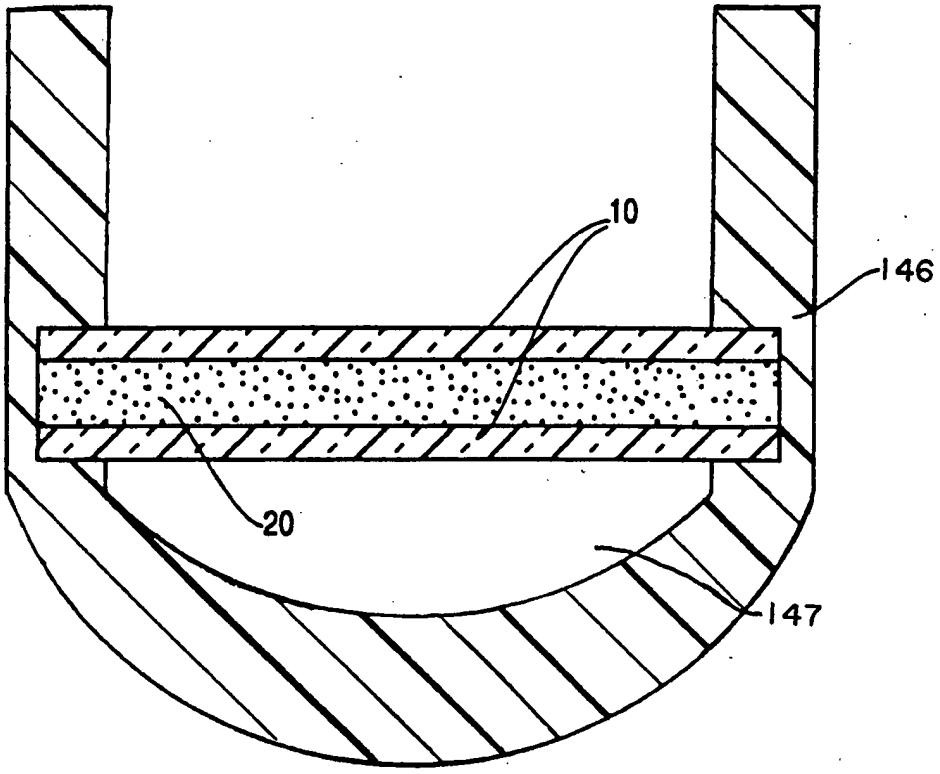


FIG. 12

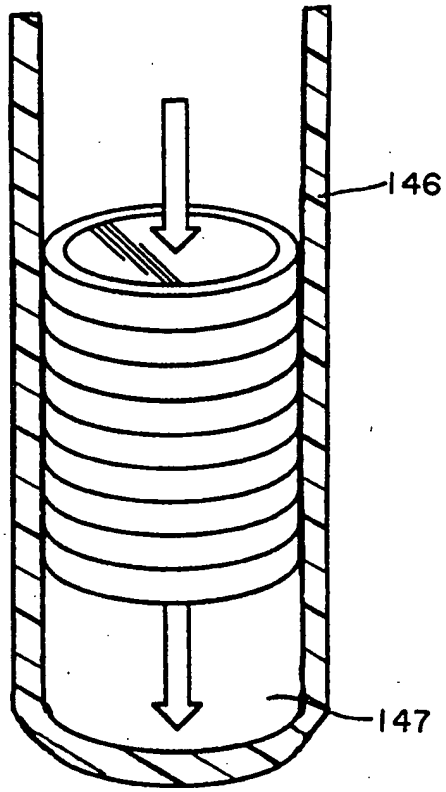


FIG. 13

FIG. 14A

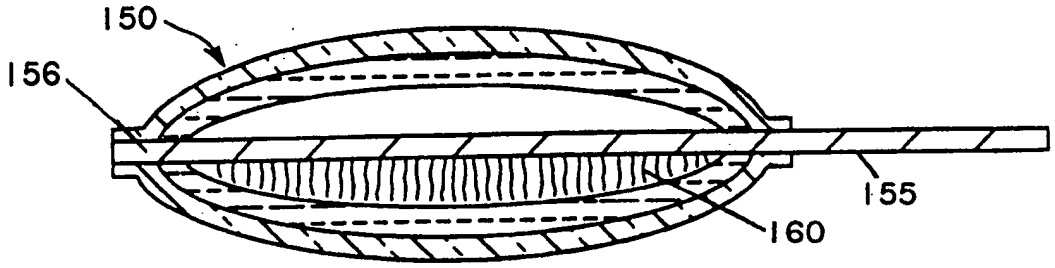
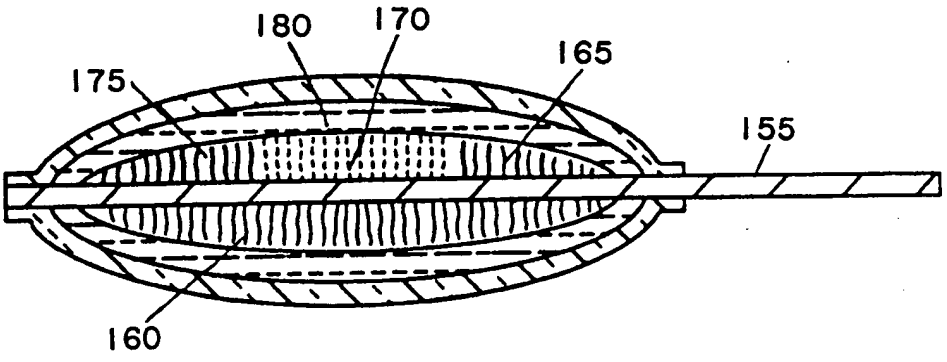


FIG. 14B



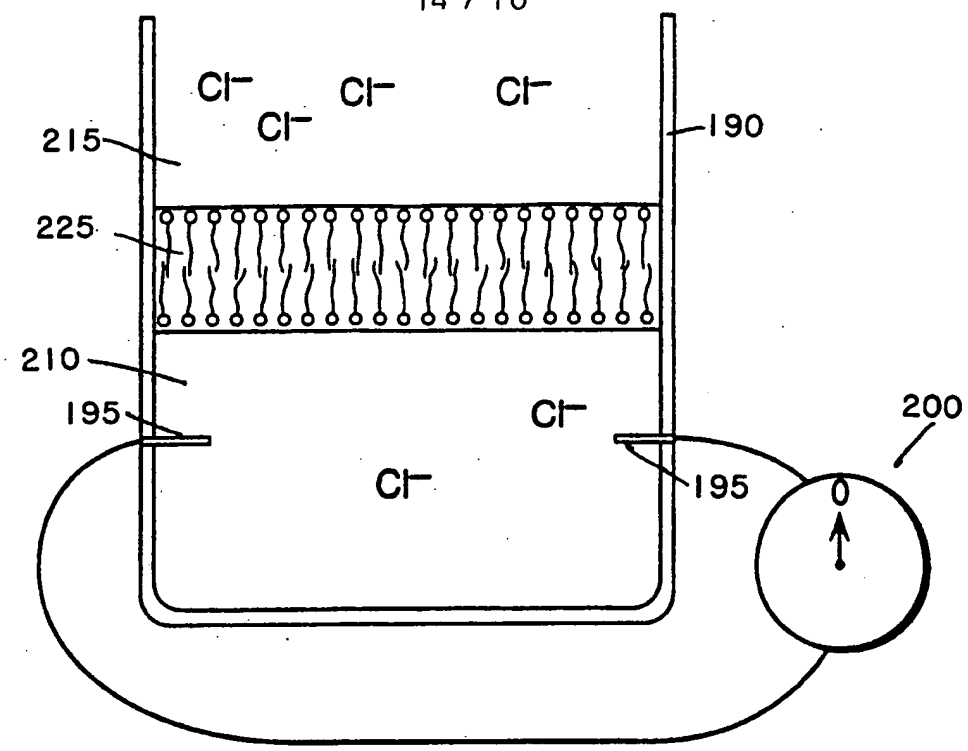


FIG. 15A

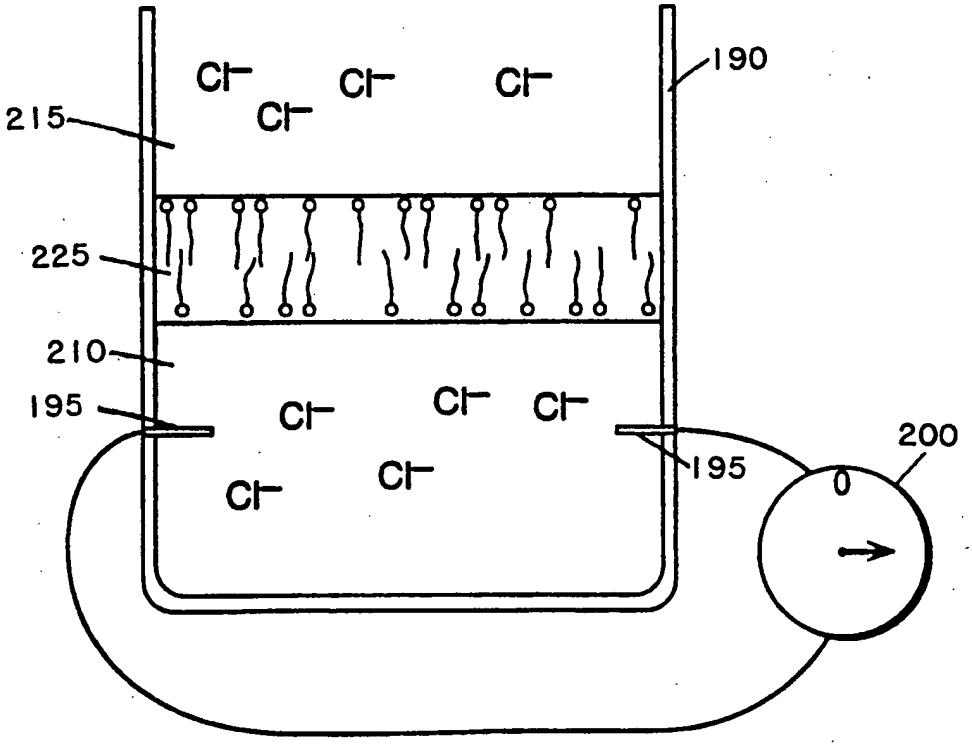


FIG. 15B

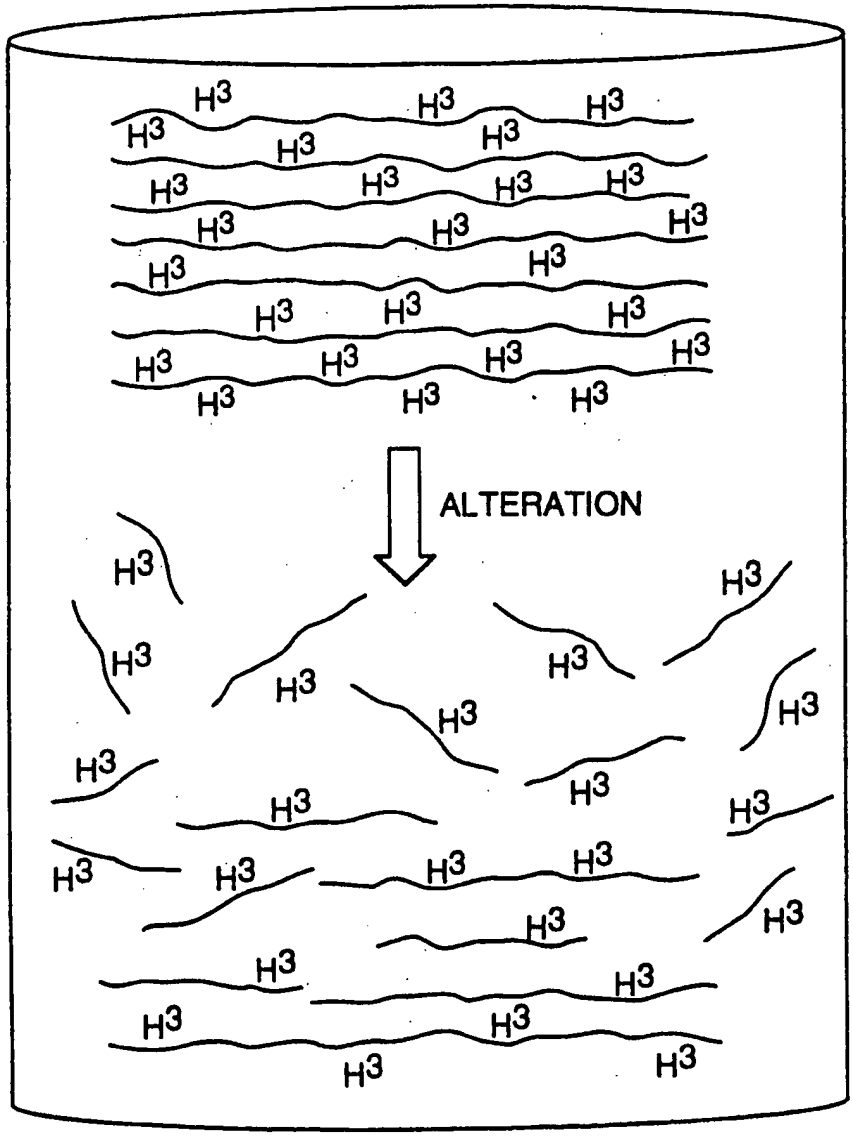


FIG. 16

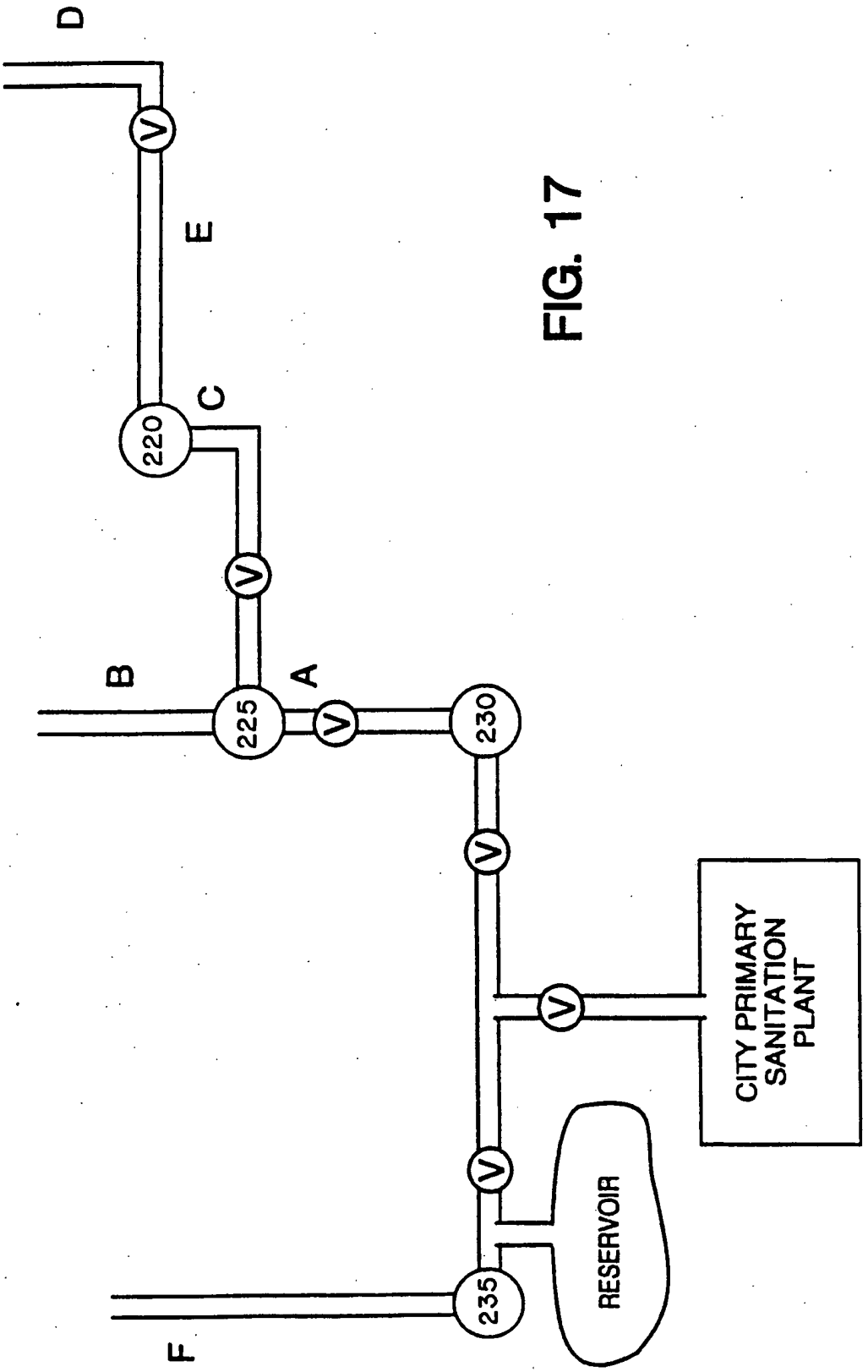

D
E
C
220
B
A
225
230
CITY PRIMARY SANITATION PLANT
RESERVOIR
235
F

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12402

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 29, 30, 32, 174, 182, 810, 817, 975; 422/57, 58, 61; 436/17, 52, 63, 172, 800, 807, 808

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, Medline, Biosis, Derwent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Annals of New York Academy of Sciences, issued 1983, Bjorn Ekwall, "Screening of Toxic Compounds in Mammalian Cell Cultures", pages 64-77, see entire document.	1-13
Y	US, A, 4,835,102 (BELL ET AL.) 30 MAY 1989, see entire document.	11-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 DECEMBER 1994	Date of mailing of the international search report 08 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Ralph Gitomer Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12402

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/00, 1/02, 1/18, 1/24; C12N 11/00, 11/04; G01N 21/00, 21/76, 31/00, 33/48, 35/08

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/4, 29, 30, 32, 174, 182, 810, 817, 975; 422/57, 58, 61; 436/17, 52, 63, 172, 800, 807, 808